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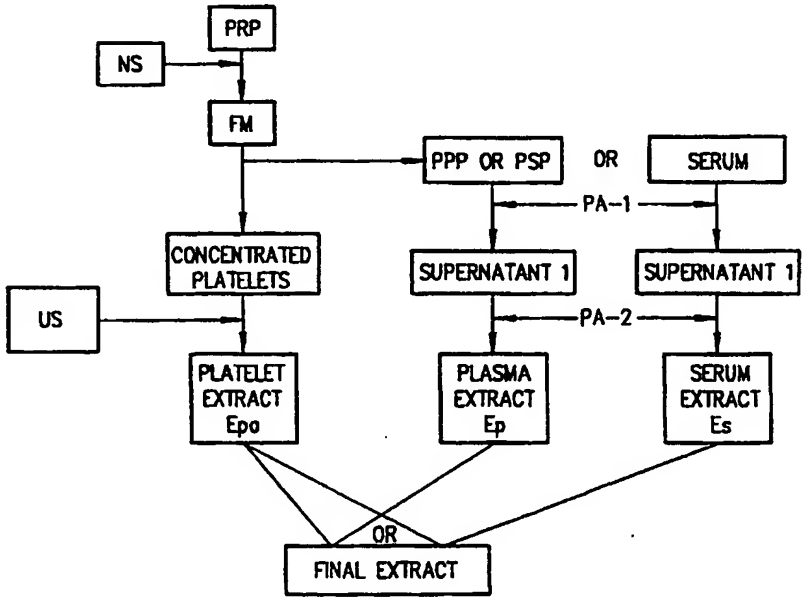
(54) Title: PROCESS FOR THE PREPARATION OF SERUM AND PLATELET GROWTH FACTORS EXTRACT

(57) Abstract

The present invention relates to a process for the preparation of platelet-serum-extract, which comprises the steps of: a) concentrating a platelet-rich-plasma prepared from whole blood by passing through a neutral support for adsorbing the platelets or by centrifuging whole blood at about 1000g to about 3000g; b) releasing the concentrated platelets of step a) by ultrasound shock; c) precipitating the concentrated plasma of step a), a platelet-poor-plasma or a serum by adding an amount of acetone solution to obtain an acetone concentration of about 68 to about 95 % at a temperature of about -15 to 5 °C; thereby obtaining a solid plasma or serum precipitate; d) the plasma or serum precipitate of step c) is reconstituted to a concentration of about 0.5 to 6 % of protein weight/volume and mixed to the released platelets of step b) to obtain a platelet-serum-extract of a concentration which substantially has a physiological concentration to give a maximum synergy in promoting wound healing. The present invention also relates to pharmaceutical composition for promoting wound healing, which comprises an effective concentration of a platelet-extract, a serum-extract or a platelet-serum-extract prepared according to the process of the present invention in association with a pharmaceutically acceptable carrier.



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PROCESS FOR THE PREPARATION OF SERUM AND PLATELET GROWTH FACTORS EXTRACT

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a process for the production of a whole blood derived serum and/or platelet extracts and their clinical uses in wound dressing.

(b) Description of Prior Art

Wound healing is a complex cascade of cellular and biochemical events which lead to wound closure and repair of tissues. Three successive phases are classically distinguished in this process:

1) the inflammatory phase, corresponding to increased vascular permeability and migration of leukocytes and macrophages;
2) the proliferative phase, characterized by fibroblast proliferation and collagen synthesis, resulting in granulation tissue formation; and
3) the remodeling phase, where collagen and granulation tissue rearrangements results in scar resorption.

The very first event that normally occurs in a wound is blood extravagation, that results in platelet aggregation and impregnation of the wound with platelet and serum constituents. Among these constituents are polypeptide growth factors, which are known to play a major role in tissue regeneration. Platelet α granules, which are released by aggregated platelets, are one of the richest physiological source of platelet-derived growth factor (PDGF) and transforming growth factor β (TGFβ), while serum contains high amounts of insulin-like growth factor I (IGF-I), IGF-II and their binding proteins (IGF-BPs) (Strovbant P & Waterfield MD, Embo. J., 1984, 2:2963-2967; Assoian RK et al., J. Biol.

Chem., 1983, 258:7155-7160; Sara VR et al., Physiol. Rev., 1990, 70:591-614).

PDGFs include PDGF, platelet derived angiogenesis factor (PDAF), TGFβ and platelet-factor-4 (PF-4), which is a chemoattractant for neutrophils (Knighton et al., *In* Growth Factors and Other Aspects of Wound Healing: Biological and Clinical Implications, Alan R. Liss, Inc., pp. 319-329 (1988)). PDGF is a mitogen and chemoattractant for fibroblasts and smooth muscle cells and is a stimulator of protein synthesis in cells of mesenchymal origin, including fibroblasts and smooth muscle cells. PDGF is also a nonmitogenic chemoattractant for endothelial cells.

TGFβ is a chemoattractant for macrophages and monocytes. Depending upon the presence or absence of other growth factors, TGFβ increases the tensile strength of healing dermal wounds. TGFβ also inhibits endothelial cell mitosis, and stimulates collagen and glycosaminoglycan synthesis by fibroblasts.

Other growth factors, such as EGF, TGFα, the HBGFs and osteogenin, are also important in wound healing. EGF, which is found in gastric secretions and saliva, and TGFα, which is made by both normal and transformed cells, are structurally related and may recognize the same receptors, which mediate cell proliferation on epithelial cells. Both factors accelerate re-epithelialization of skin wounds.

The *in vivo* mode of action of these growth factors involves chemoattraction at the wound site, cell proliferation and collagen synthesis. One very interesting feature of these products is that some of them, namely PDGF and IGFs, work synergistically in stimulating wound repair (Lynch SE et al., J. Clin. Invest., 1989, 84:640-646; Greenhalgh DG et al., Wound. Rep. Reg., 1993, 1:54-62).

Growth factors are, therefore, potentially useful for specifically promoting wound healing and tissue repair. The addition of exogenous growth factors to a wound has been shown to increase the rate at which the wound is closed, the number of cells in the healing area, the growth of blood vessels, the total rate of deposition of collagen, and strength of the scar (Carter et al., in Growth Factors and Other Aspects of Wound Healing: Biological and Clinical Implications, Alan R. Liss, Inc., pp. 303-317 (1988)). Platelet-derived wound healing formula (PDWHF), a platelet extract which is in the form of a salve or ointment for topical application, has been described by Knighton et al. (Ann. Surg., 1986, 204: 322-330).

Knighton et al. (Ann. Surg., 1986, 204: 322-330 & *In* Growth Factors and Other Aspects of Wound Healing: Biological and Clinical Implications, Alan R. Liss, Inc., pp. 319-329 (1988)) describe a process for the preparation of a platelet-derived wound healing composition by centrifuging platelets and treating them with thrombin to stimulate the production of a releasate, which can be combined with collagen. Knighton et al. obtain a platelet-derived releasate having a concentration of growth factors of about 10 to about 17%, which is not practically usable. Also, this platelet-derived releasate contains essentially denatured or non-mature growth factors due to the use of thrombin as a chemical shock to break apart the platelets and release their content.

Surgical adhesives and tissue sealants which contain plasma proteins are known and are used for sealing internal and external wounds in order to reduce blood loss and maintain hemostasis. Such sealants typically contain blood clotting factors and other blood proteins.

Stroetmann, U.S. Patent Nos. 4,427,650 and 4,427,651, describes the preparation of an enriched plasma derivative in the form of a powder that contains fibrinogen, thrombin and/or prothrombin, and a fibrinolysis inhibitor, and may also contain other ingredients, such as a platelet extract.

Clinical evaluation of recombinant growth factors are underway but it remains so far uncertain whether the therapeutic benefit of these drugs will pay for their high cost of production specially if used in combination.

Autologous human platelet derived wound healing formula, made of thrombin activated platelet α granules, has also been shown to induce the healing of chronic ulcers, thus indicating that growth factor extracts may constitute an advantageous alternative to the use of recombinant growth factors if proven to be economically extractable.

In addition, improved fibrin glue delivery systems have been disclosed (Miller et al., U.S. Patent No. 4,932,942; Morse et al., PCT Application WO 91/09641).

Fibrin glue, which is also called fibrin sealant, is primarily formulated for clinical topical application and is used to control bleeding and promote wound healing. The clinical uses of fibrin glue have recently been reviewed (Gibble et al., Transfusion, 1990, 30:741-747; Lerner et al., J. Surg. Res., 1990, 48:165-181). Fibrin glues are commercially available. For example, IMMUNO AG (Vienna, Austria) and BEHRINGWERKE AG (Germany) presently have such products on the market (U.S. Patent Nos. 4,377,572 and 4,298,598).

Fibrin glues are prepared from plasma. The precise components of each fibrin glue are a function of the particular plasma fraction used as a starting

material. Typically fibrin glue contains a mixture of proteins that, upon mixing with thrombin, form a clot. For example, fibrin glue can be prepared from plasma by cryoprecipitation followed by fractionation, to yield a composition that forms a sealant or clot upon mixture with thrombin or an activator of thrombin. Fractionation of plasma components can be effected by standard protein purification methods, such as ethanol, polyethylene glycol, and ammonium sulfate precipitation, and ion exchange, and gel filtration chromatography.

Fibrin glues generally include a fibrinogen concentrate, which contains fibronectin, Factor XIII, von Willebrand factor, and dried human or bovine thrombin. It is prepared in lyophilized form and is mixed with a solution of calcium chloride immediately prior to use. Upon mixing, the components coagulate on the tissue surface and form a clot that includes cross-linked fibrin. Factor XIII, which is present in the fibrinogen concentrate, catalyzes the cross-linking, fibrin glue, by sealing tissues face to face, prevents air or fluid leaks, and thereby induces hemostasis. By virtue of the ability to maintain hemostasis and reduce blood loss, fibrin glue promotes wound healing. It does no, however possess true wound healing properties. Because fibrin glue is suitable for both internal and external injuries and is useful to maintain hemostasis, it would be desirable to enhance its wound healing properties.

It would be highly desirable to be provided with a economical and easy to carry out process for the isolation of contamination-free blood derived serum/platelet extract for use in a wound dressing composition. Such a blood derived serum/platelet extract should be absolutely free of Hepatitis B and HIV contaminants.

It would be highly desirable to be provided with a process for the isolation of whole blood derived serum/platelet extract containing non-denatured growth factors at a concentration of about 54 to about 99.8%, which extract is suitable for use in a wound dressing composition.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide for an easy process for the isolation of whole blood derived serum/platelet extract containing non-denatured growth factors at a high concentration of about 54 to about 99.8% , which extract is suitable for use in a wound dressing composition.

Another aim of the present invention is to provide for a process for the production of growth factors extracts nearly 100% identical to their human counterparts.

Another aim of the present invention is to provide a blood derived serum/platelet extract for use in a wound dressing composition being characterized by a total absence of potential viral transmission of human origin (Hepatitis, HIV, etc.).

A further aim of the present invention is to provide for pharmaceutical compositions for promoting wound healing.

Surprisingly and in accordance with the present invention there is provided a process for the preparation of platelet-serum-extract, wherein the growth factors are substantially non-denatured and in high concentration essentially due to the new use of ultrasound as a shock treatment. The novel process in accordance with the present invention comprises the steps of:

a) concentrating a platelet-rich-plasma prepared from whole blood by passing through a neutral support for adsorbing the platelets or by centrifuging whole blood at about 1000g to about 3000g, preferably at 1500g;

b) releasing the concentrated platelets of step a) by ultra-sound shock;

c) precipitating the concentrated plasma of step a), a platelet-poor-plasma or a serum by adding an amount of acetone solution to obtain an acetone concentration of about 68 to about 95% at a temperature of about -15 to 5°C; thereby obtaining a solid plasma or serum precipitate;

d) the plasma or serum precipitate of step c) is reconstituted to a concentration of about 0.5 to 6% of protein weight/volume and mixed to the released platelets of step b) to obtain a platelet-serum-extract of a concentration which substantially has a physiological concentration to give a maximum synergy in promoting wound healing.

In accordance with the present invention, the preferred wound dressing includes a combination of the main growth factors present at the wound site, i.e. PDGF, TGFβs and IGFs.

In accordance with one embodiment of the present invention, a process for the production of a whole blood derived serum/platelet extract is described along with its biological potencies as a wound healing enhancer when associated with a fibrin matrix and/or biodegradable gelifying matrix.

In accordance with one embodiment of the present invention, the whole blood derived serum/platelet extract characterized by a total absence of potential viral transmission of human origin (Hepatitis, HIV,

etc.), may be prepared from decontaminated human whole blood or whole blood of porcine or bovine origin or of other closely related species.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a reaction scheme of a process in accordance with one embodiment of the present invention;

Fig. 2 is a Western blot for the identification of IGFBPs in serum, platelet and serum/platelet extracts;

Fig. 3 is a SDS-PAGE analysis of serum, platelet and serum/platelet extracts;

Fig. 4 illustrates the effect of a serum/platelet extract (PSE) on a MTT test of human fibroblast survival;

Fig. 5 illustrates the wound breaking strength of (A) control vs fibrin glue-treated rats (experiment 1) and of (B) control vs PSE-treated rats in presence or absence of fibrin glue (experiment 2);

Fig. 6 illustrates the dose-effect relationship of platelet/serum extracts (PSE) on wound breaking strength in rats; and

Fig. 7 illustrates the morphometric analysis of 14 day-old wounds treated with PSE alone, PSE in fibrin vs control wounds.

DETAILED DESCRIPTION OF THE INVENTION

With reference to the drawings, it will be seen that Fig. 1, which illustrates the reaction scheme of a process in accordance with one embodiment of the present invention. The terms referred therein are defined as follows:

"PRP" is a platelet-rich-plasma, preferably of porcine origin prepared by low speed centrifugation in order to pellet and remove erythrocytes and leukocytes.

"PE" is a platelet extract.

"NS" is a mono- or poly-atomic neutral support, having an amorphous structure or a crystalline lattice (with or without defect) selected from the following polygons, but not limited to this list: hexagon, cubic, mono- or tri-clinic, romboedric and orthorhombic. Depending on the exact procedure used, the support may be of carbon or heteroatomic matter. It may have a single or several anomeric phases and may include also in its network one or more of the following heteroatomes: cobalt, Nickel, sulfur, oxygen, tungsten, aluminum, silicon, nitrogen, fluorine among others. The preferred support is graphite powder or activated carbon.

"FM" is a neutral filtering membrane especially designed using a non-soluble and hydrophobic organic polymer. This membrane offers a wide filtering surface which confers its almost non-saturable filtering capacity.

"US" is representing ultra-sound shock.

"PPP" or "PSP" is a platelet-poor-plasma resulting from a unique passage of support-adsorbed-platelet containing plasma through the filtering membrane.

"PA-1" and "PA-2" are each respectively precipitating agent 1 and 2 of the following chemical formulas:

$$Q\text{-}H \quad \text{or} \quad M_xY_n$$

wherein:

Q is a radical homo- or hetero-cyclic, alkyl, silyl, cycloalkyl, alkenyl, aryl, aralkyl including at least one functional group

selected from the following, but not limited to this list, acid, alcohol, amine, amide, aldehyde, ketone, enol, oxime, halide, imine, enamine, ester, ether, phosphite, phosphate, nitro, nitroso, sulfamide, sulfate, sulfite, sulfoxyde, sulfone, thiosulfone, thioether, and nitrile;

M is an ammonium or phosphonium ion or any positive ion resulting from the chemical ionisation of elements of groups 1 to 3 of the Periodical Table of Mendéléev;

Y is a carboxylate, a sulfate, a nitrate, a halide, a hexafluorophosphate, or a tetrafluoroborate ion, or any stable anion resulting from the Lewis acid-base interaction; and

x and n are identical or different and are any number between 1 and 6.

The precipitating agents PA-1 and PA-2 may be used as such or combined together in a mixture with one or more agents, preferably in aqueous suspension or solution of a pH range from 1 to 12.

The preferred precipitating agent PA-1 is an amount of acetone solution where the resulting acetone concentration is about 68 to about 72%.

The preferred precipitating agent PA-2 is an amount of acetone solution where the resulting acetone concentration is about 84 to about 95%.

The process of the present invention excludes from the platelet-rich-plasma (PRP) step any manipulation or rinsing of the free platelets by successive centrifugation. In accordance with the process of the present invention, there is no need for the expensive intermediates of the prior art, such as thrombin.

The first step of the process of the present invention consists mainly in the preparation of the platelet extract (PE) based on the use of ultrasound as a shock treatment for the release of the material of filtered platelet. In addition to its low cost, this step is simple and can be carried out in less than two hours, even using large quantities of material.

The second step of the process of the present invention consists mainly in the preparation of the serum extract (SE) while overcoming the drawbacks of the prior art procedures. This step allows for the chemical transformation of a platelet-poor-plasma or of a serum in a hyperactive growth factor concentrate of molecular weight between 0 and 50 kDa.

The platelet extract (PE) and the serum extract (SE) may be prepared in advance or in parallel and may be stored and reconstituted in water or in any appropriate physiological buffer such as saline, phosphosaline, Krebs or Hepes.

The preferred embodiments of the process of the present invention are more specifically described as follows, which is intended to illustrate the invention rather than to limit its scope.

1. BLOOD EXTRACTS PREPARATION

1.1 Blood collection

For experimental purposes, porcine whole blood was used in accordance with the present invention due to its availability and its contamination-free characteristic. Thus, young Landrace, Yorkshire, male castrated piglets (2 month-old, 15kg B.W. were purchased from a local breeder). They were housed for 2-3 days in our facilities with free access to water and food. For blood collection, they were anaesthetized with ketamine hydrochloride (10 mg/kg BW), then with

fluothane by intratracheal administration. A cannula was aseptically inserted into one carotid artery and animals were exsanguinated. Blood was collected in 20% acid-citrate-dextrose (ACD) buffer (8 g citric acid monohydrate; 22 g dextrose; 26 g sodium citrate dihydrate in one liter of picopure distillated water, >18.2 megaohms). When serum was needed, blood was collected without any anticoagulant buffer.

1.2 Platelets extract (PE)

1.2.1 General procedure

Whole ACD blood was collected in 20% acid-citrate-dextrose (ACD) buffer and centrifuged for about 3 min. at 1500g (BRAKE=MAXIMUM; Rotor™ JA10; Beckman™ J2-21). The supernatant, platelet-rich-plasma (PRP), was collected, its volume was calculated and the platelet's count was effected using a Coulter Counter ZM™ (Coulter Electronics of Canada, Ltd).

1.2.2 Procedure with solid support and shock treatment

The collected platelet-rich-plasma (PRP) was immediately mixed with synthetic activated carbon (No. 24,227; Aldrich Chemical Co, MW) that had been previously soaked in picopure distilled water. The amount of graphite to add was determined using the following general formula I:

$$Q = k.[PT].\ V.\ 10^{-9}, \qquad I$$

where Q is the amount of activated carbon to add (g), k is an empirically determined constant (between 0.01 and 0.80, preferably 0.082), [PT] is the platelet concentration per liter of PRP and V the PRP volume (L). Activated carbon was used as a neutral support to selectively adsorb platelets. Other supports, such as

silicon nitride, silicon carbide, or activated carbon G-60™ (Aldrich) or Carbon Decolorizing Alkaline™ (Norit A, Fischer Scientific, Nepean, Ont.) were also successfully used.

The mixture activated carbon-PRP was gently mixed for 2 min., then filtered through a Whatman folded filter paper 2V™ (Whatman International Ltd, England). The filter, containing platelets and activated carbon, was washed twice with phosphosaline buffer (PBS, pH 7.0) in order to completely eliminate proteins from serum. The content of the filter was then transferred into a glass vial containing picopure distilled water.

The vial was subjected to a shock treatment consisting of either heating at 100°C for one min. and then cooled at 25°C or of two ultra-sound treatment with a sonicator (Kontes™ 40-watt; output control:60; monitor scale:20) for about 5 sec. to 10 min. with a pause of 30 sec. in between.

The mixture was finally centrifuged at 2,000 to 10,000g for 5 min., the activated carbon pellet was reconstituted in picopure water, centrifuged, and both supernatants were pooled and lyophilized. The resulting powder was the platelet extract (PE).

1.2.3 Procedure with shock treatment and no solid support

The collected platelet-rich-plasma (PRP) was centrifuged for about 20 min. at 1500g (BRAKE=6; Rotor™ JA10; Beckman™ J2-21). The precipitate, corresponding to the platelets, was resuspended in phosphate buffer saline (PBS) to obtain a final concentration of 10^9 platelet equivalent per ml (pq/ml), which corresponds to the first washing step.

The resuspended platelets were centrifuged for a second time at the conditions mentioned above. The

precipitated platelets were resuspended in picopure, distilled water to a final concentration of 10^{10} platelet equivalent per ml (pq/ml).

The solution was cooled to about -5 to about 4°C in a ice bath and was subjected to a shock treatment consisting of two ultra-sound treatment with a sonicator (Kontes™ 40-watt; output control:60; monitor scale:20) for 45 sec. with a pause of 30 sec. in between.

The treated solution was frozen using liquid nitrogen and lyophilized overnight. The lyophilized platelets were reconstituted in picopure water, centrifuged, and both supernatants were pooled and lyophilized. The resulting powder was the platelet extract (PE).

1.3 Serum extract (SE)

A serum extract was prepared by double acetone protein precipitation in acidic conditions. Whole serum was first acidified with formic acid (88% formic acid, Fisher Scientific, Neapan, Ontario) to pH 1.9-2.0, then mixed with acetone (Anachemia, Montreal, Canada) to a final concentration of about 68% to about 72% acetone, the preferred acetone concentration being 70%. The mixture was filtered through a Whatman filter paper 2V™. The filtrate was collected and acetone was added to a final concentration of about 84% to about 95%, the preferred final concentration being 90%. The mixture was stirred up and again filtered through a Whatman filter paper 2V™. The filter content was washed with pure acetone, then with ether in order to completely remove acetone. Ether was air-evaporated, the final powder was reconstituted in picopure water (>18.2 megaohms) (1% w/v), filter-sterilized through

0.2 µm filters (Nalgene, Rochester, NY) and kept frozen at -75 to about -85°C.

The serum precipitate may be reconstituted to a concentration of about 0.5 to 6% of protein weight/volume, preferably to a concentration of about 1 to 2% of protein weight/volume (for example, 10 mg/ml=1.0%).

1.4 Combination of platelet and serum extracts

For all *in vivo* studies, the PE was reconstituted with the 1% SE to a final concentration of 10^{10} platelet equivalent per ml. The mixture was centrifuged at 5000 g for 15 min. at +4°C, then filter sterilized through 0.2. µm filters and either stored at -75°C or lyophilized. The final product was termed platelet-serum-extract (PSE). For the need of analytical and some *in vitro* studies, the PE was reconstituted in picopure water (>18.2 megaohms) (10^{10} platelet equivalent/ml).

2. ANALYTICAL PROCEDURES

Proteins were measured in PE, SE and PSE by a Lowry method (J. Biol. Chem., 1951, 193:265-275) using a commercial kit sold by Sigma Chemical (St Louis, MO).

Platelet-derived growth factor (PDGF) was measured in PSE by radioimmunoassay using a commercial kit (Amersham International, UK) according to the following procedure.

PORCINE PDGF RIA PROCEDURE

Tube #	Standard or sample	Buffer	I^{125}-PDGF-2 ββ	Ab_1 Raised in goat
1- Trace	–	–	100 µl	–
2- Background	–	400 µl	100 µl	–
3- Zero	–	300 µl	100 µl	100 µl
4- 0.044 ng/tube	100 µl	200 µl	100 µl	100 µl
5- 0.137 ng/tube	100 µl	200 µl	100 µl	100 µl
6- 0.410 ng/tube	100 µl	200 µl	100 µl	100 µl
7- 1.230 ng/tube	100 µl	200 µl	100 µl	100 µl
8- 3.700 ng/tube	100 µl	200 µl	100 µl	100 µl
9- 11.000 ng/tube	100 µl	200 µl	100 µl	100 µl
10- 33.000 ng/tube	100 µl	200 µl	100 µl	100 µl
11-100.000 ng/tube	100 µl	200 µl	100 µl	100 µl
12- unknown ng/tube	100 µl	200 µl	100 µl	100 µl

Incubation: 20 to 24 hours at 22°C.

Precipitation: 2nd antibody precipitation (100 µl)

50 µl 4% normal goat plasma and 50 µl donkey anti-goat gamma globulins (diluted 1 in 5 with buffer), vortex, leave 18 hrs or overnight at 22°C, centrifuge 35 mins at 3200 rpm, decant and count

Dilution of standard: Porcine PDGF 1 ng/ml frozen in aliquot of 500 ml (500 ng/500 µl)

1. 200 µl is added to 400 µl buffer = 33.3 ng/100 µl;
2. A serial dilution is made by 1/3 for standard from 100 ng/100 µl to 0.044 ng/100 µl.

Dilution of Ab_1 (anti-hPDGF-BB, AB-220-NA):

1. Frozen in 40 µl aliquots of a dilution of 1/1;
2. 20 µl in 1980 ml of buffer make a dilution of 1/100. Take 100 µl of 1/100 Ab_1 in 16 µl buffer, make a final dilution of 1/80K.

Tracer: ^{125}I-$PDGF_1$ (Dupont) diluted in PDGF buffer so that 100 µl = 13 000 cpm.

According to the manufacturer's data, cross reactivity of porcine PDGF is 38% with the anti human PDGF antibody used in the kit. Results were therefore converted in porcine PDGF equivalent by dividing them by 0.38.

Transforming growth factor β1 and β2 were measured in PSE using the Quantikine test™ commercially available (Amersham International, V.K.). Before measurement, extracts were mixed 1:2 either in distilled water or in trifluoroacetic acid (TFA) 1%, incubated for 1 hour at room temperature, lyophilized and reconstituted with water to their initial volume. This procedure was conducted in order to determine if TGFβ was present in extracts as an active or latent, high molecular weight form.

Insulin-like growth factor-I and II (IGFI and II) were measured in SE by radioimmunoassay after formic acid-acetone extraction (Bowsher R.R. et al., Endocrinology, 1991, 128:805-814). Briefly, SE (100 µl) was acidified with formic acid 8.0 M, containing 0.5% Tween 20™ (Biorad Lab., Richmond, CA), then precipitated with acetone (350 µl). The supernatant was then diluted in RIA buffer for IGFs measurements (1/60 for IGF-I and 1/300 for IGF-II, in a 30 mM phosphate buffer, pH 7.4, containing 0.02% protamine sulfate, grade II, 10 mM EDTA, 0.02% sodium azide and 0.25% bovine serum albumin RIA grade, all buffer components from Sigma Chemicals, St Louis, MO).

IGF-I was then measured in neutralized extracts by double antibody RIA using recombinant human IGF-I (Bachem California) for iodination and standards, and polyclonal anti-hIGF-I antiserum kindly provided by Dr. Underwood (University of North Carolina) through the National and Pituitary Program of the NIDDK, as previously described by Brazeau P. and Abribat T. (75th Ann. Meet. of the Endocrine Soc., Las Vegas, NA, June 9-12, 1993, Abst. 1327) according to the following procedure.

hIGF-I RIA PROCEDURE

Tube #	Standard or sample	IGF-I Buffer	I^{125}-RIGF-1	Ab_1 Raised in rabbit
1- Trace	–	–	100 µl	–
2- Background	–	400 µl	100 µl	–
3- Zero	–	300 µl	100 µl	100 µl
4- 15.6 pg/tube	100 µl	200 µl	100 µl	100 µl
5- 31.2 pg/tube	100 µl	200 µl	100 µl	100 µl
6- 62.5 pg/tube	100 µl	200 µl	100 µl	100 µl
7- 125 pg/tube	100 µl	200 µl	100 µl	100 µl
8- 200 pg/tube	100 µl	200 µl	100 µl	100 µl
9- 500 pg/tube	100 µl	200 µl	100 µl	100 µl
10-1000 pg/tube	100 µl	200 µl	100 µl	100 µl
11-2000 pg/tube	100 µl	200 µl	100 µl	100 µl
12- unknown pg/tube	100 µl	200 µl	100 µl	100 µl

Incubation: 20 to 24 hours at 4°C.

Precipitation: 2nd antibody precipitation (100 µl)

50 µl 4% normal rabbit plasma and 50 µl goat anti-rabbit gamma globulins (diluted 1 in 5 with buffer), vortex, leave 18 hrs or overnight at 4°C, centrifuge 35 mins at 3200 rpm, decant and count.

Dilution of standard: Human IGF-I 100 µg/ml frozen in aliquot of 10 µl (1 µg/10 µl)

1. 10 µl is added to 990 µl buffer = 100 000 pg/100 µl;
2. 50 µl is added to 950 µl buffer = 5000 pg/100 µl;
3. 400 µl is added to 600 µl buffer = 2000 pg/100 µl;
4. A serial dilution is made for standards from 2000 to 15.6 pg/100 µl.

Dilution of Ab_1 (Gropep):

1. Frozen in 50 µl aliquots of a 1/100 dilution;
2. 50 µl in 15 ml IGF-I buffer (final dilution for RIA 1/150K)

Tracer: ^{125}I-RIGF-I diluted in IGF-I buffer so that 100 µl = 10 000 to 11 000 cpm.

IGF-II was also measured in neutralized extracts in the same RIA buffer by double antibody RIA (Brazeau P. and Abribat T., 75th Ann. Meet. of the Endocrine Soc., Las Vegas, NA, June 9-12, 1993, Abst 1327) using recombinant human IGF-II (Bachem California) for iodination

and standards, and monoclonal anti hIGF-II antibodies diluted 1/50 K (Amano International Enzymes Co) according to the following procedure.

hIGF-II RIA PROCEDURE

Tube #	Standard or sample	IGF-II Buffer	I^{125}- hIGF-II	Ab_1 Raised in rabbit
1- Trace	–	–	100 µl	–
2- Background	–	400 µl	100 µl	–
3- Zero	–	300 µl	100 µl	100 µl
4- 3.9 pg/tube	100 µl	200 µl	100 µl	100 µl
5- 7.8 pg/tube	100 µl	200 µl	100 µl	100 µl
6- 15.6 pg/tube	100 µl	200 µl	100 µl	100 µl
7- 31.2 pg/tube	100 µl	200 µl	100 µl	100 µl
8- 62.5 pg/tube	100 µl	200 µl	100 µl	100 µl
9- 125 pg/tube	100 µl	200 µl	100 µl	100 µl
10- 250 pg/tube	100 µl	200 µl	100 µl	100 µl
11- 500 pg/tube	100 µl	200 µl	100 µl	100 µl
12- unknown pg/tube	100 µl	200 µl	100 µl	100 µl

Incubation: 20 to 24 hours at 4°C.

Precipitation: 2nd antibody precipitation (100 µl)

50 µl 4% normal mouse plasma and 50 µl goat anti-mouse gamma globulins (diluted 1 in 5 with buffer), vortex, leave 18 hrs or overnight at 4°C, centrifuge 35 mins at 3200 rpm, decant and count

Dilution of standard: Human IGF-II 100 µg/ml frozen in aliquot of 10 µl (1 µg/10 µl)

1. 10 µl is added to 500 µl buffer = 200 000 pg/100 µl;
2. 50 µl is added to 1950 µl buffer = 5000 pg/100 µl;
3. 100 µl is added to 900 µl buffer = 500 pg/100 µl;
4. A serial dilution is made for standards from 500 to 3.9 pg/100 µl.

Dilution of Ab_1:

1. Frozen in 50 µl aliquots of a 1/10 dilution;
2. 50 µl in 50 ml IGF-II buffer (final dilution for RIA 1/50K)

Tracer: ^{125}I-IGF-II diluted in IGF-II buffer so that 100 µl = 10 000 to 11 000 cpm.

Cross reactivity of IGF-II in the IGF-I RIA was 0.2% and cross reactivity of IGF-I in the IGF-II RIA

was 3.5%. ED_{50} from standard curves were typically 70 pg/tube and 90 pg/tube for IGF-I and IGF-II RIAS, respectively.

IGF-binding proteins (IGFBP) were measured in PE, SE and PSE by a western ligand blotting methodology, using ^{125}I-IGF-I as radioligand, adapted from the procedure originally described by Hossenlopp et al. (Anal. Biochem., 1986, 154:138-143). Non reduced samples were subjected to SDS-PAGE electrophoresis on 12% polyacrylamide gel slabs, then electrotransferred onto nitrocellulose. Air dried nitrocellulose sheets were sequentially incubated for 30 min. in Tris-buffered saline (TBS) containing 3% Nonidet™ P-40 (Calbiochem Co, La Jolla, CA), for 2h in TBS-1% BSA, and finally for 10 min. in TBS-0.1% Tween 20™. Sheets were transferred into sealed plastic bags containing 1.5. 10^6 cpm ^{125}I-IGF-I in 20 ml. After an overnight incubation with continual gentle agitation, sheets were washed twice in TBS-0.1% Tween 20™, then 3 times in TBS, and finally autoradiographed at -70°C on X-ray films for 48 h. For quantitative analysis, bands of the nitrocellulose sheets corresponding to those on autoradiograms were cut and counted in a gamma counter.

Growth factors and protein content were measured in a series of platelet and serum extracts (PE, SE and PSE).

In PE, PDGF levels averaged 3.5 ± 0.5 ng/ml (mean ± SEM of 7 measurements).

In PE, using the ultra-sound as a shock treatment, PDGF levels unexpectedly varied from 3.16 to 14.2 µg/l.

In four PE, non extracted (water extracted) TGF β 2 content was 4145 ± 542 pg/ml, and acid extracted TGFβ 2 content was identical (4169 ± 489 pg/ml) sug-

gesting that TGFβ 2 was entirely present in PE in its active form of about 25 KD.

In PE, using the ultra-sound as a shock treatment, TGFβ 1 levels unexpectedly varied from 0.873 to 3.9 µg/l.

In PE, using the ultra-sound as a shock treatment, TGFβ 2 levels unexpectedly varied from 5.8 to 46.0 ng/l.

In SE, IGF-I and IGF-II concentrations averaged 200 ± 16 ng/ml and 452 ± 82 ng/ml respectively (mean ± SEM of 7 measurements). As shown in Fig.2, IGFBPs could be detected both in SE and PSE but not in PE. The major IGFBP in serum, IGFBP3 (doublet migrating at 37 and 41 KD), was completely absent in serum extracts, while the three other IGFBPs in serum (IGFBP2, 1 and 4, migrating at 31, 27, and 22 KD respectively) were found in SE and PSE, at a concentration of 15-25% that in serum.

In SE, using the ultra-sound as a shock treatment, IGF-I concentration unexpectedly varied from 16.9 to 88 µg/l and IGF-II concentration varied from 47.9 to 356 µg/l.

In SE, using the ultra-sound as a shock treatment, TGFβ 1 level unexpectedly varied from 1.98 to 21.5 µg/l and TGFβ 2 level varied from 0.027 to 0.290 µg/l.

Total protein content was 5.3 ± 0.5 mg/ml in PE (mean ± SEM of five determinations), 6.3 ± 0.8 mg/ml in SE (mean ± SEM of five determinations) and 12.2 ± 0.8 mg/ml in PSE (mean ± SEM of six determinations).

Finally, a series of PE, SE and PSE were subjected to a SDS-PAGE electrophoresis on 12% polyacrylamide gels, and silver-stained according to the method described by Morissey JH (Anal. Biochem., 1981, 117:307-310).

A protein staining of typical PE, SE and PSE is shown in Fig. 3. The major contaminant in PSE was identified as a 50-55 KD weighing group of proteins of seric origin.

3. *IN VITRO* ASSAYS

3.1 *In vitro* assays porcine primary cultured fibroblasts

In vitro bioassays were performed using porcine primary cultured fibroblasts (PPCF) provided by our animal facilities (Centre de Recherche Louis Charles Simard, Hôpital Notre-Dame, Montréal, Canada). Cells were cultured in DMEM (Cat.#11885; Gibco BRL, Burlington, Ontario) containing 10% characterized Fetal Calf Serum (FCS, Hyclone Lab. Inc., Logan, UT) and 1% penicillin-streptomycin (Cat.#600-5140; Gibco BRL, Burlington, Ontario) for assays. All cell incubations were performed in temperature and air-controlled incubators (37°C, 100% humidity and 5% CO_2).

3.1.1 Proliferation assay for porcine primary cultured fibroblasts

Cells were plated in 12-well-plates in containing 10% FCS and 1% penicillin-streptomycin at a density of 80 x 10^3 cells in one ml per well. Twenty four hour later, medium was replaced by a test medium (DMEM, 0.5% FCS and 1% penicillin-streptomycin) containing test samples (0 to 200 µl/ml SE, PE or PSE; 0 to 100 ng/ml porcine PDGF-R & D system, MN, Cat.#125PD). Maximal stimulation of proliferation was evaluated by adding 10% FCS in control wells. Each dose of each test sample was assayed in triplicate.

Two days later, cells were washed with DMEM, then trypsinized with 1 ml Trypsine/EDTA 1 x (Gibco, Cat.#25-300-013). Trypsinisation was stopped with 100 µl of pure FCS, cells in suspension were triturated,

diluted. Finally, they were counted in a Coulter Counter ZM™ (Coulter Electronics of Canada, Ltd), equipped with a Coulter Channelyzer 256™, allowing for measurement of both cell number and cell average diameter.

The effect of PE, SE and PSE on cell proliferation was determined using a porcine primary cultured fibroblasts (PPCF).

In this cell line, both PE and SE stimulated cell proliferation in a dose-dependent manner between 1 and 100 µl of extracts. Combination of PE and SE was synergic on cell proliferation, that is the effect of PSE was more than the addition of the effects of PE and SE. This synergy was observed at the doses of 1 and 10 µl/ml, but not 100 µl/ml. In fact, the 100 µl/ml dose was less potent than the 10 µl/ml dose in all PSE tested. A dose response study from 0 to 200 µl/ml revealed that PSE induced a dose-related linear stimulation of cell proliferation from 0 to 10 µl/ml, the effect being progressively blunted between 50 and 200 µl/ml. Thus, the maximal effect on cell proliferation was estimated to be at 10-50 µl/ml of PSE.

3.2 *In vitro* assays for 3T3-Swiss albino cells

In vitro bioassays were performed using 3T3-Swiss albino cells (ATCC No. CCL 92, American Culture Type Collection, Rockville, Maryland, U.S.A.). Cells were cultured in RPMI 1640™ (Cat.#430-3400EB, Gibco BRL, Burlington, Ontario) containing 10% characterized Fetal Calf Serum (FCS, Hyclone Lab. Inc., Logan, UT) and 1% penicillin-streptomycin (Cat.#600-5140; Gibco BRL, Burlington, Ontario) for assays. All cell incubations were performed in temperature and air-controlled incubators (37°C, 100% humidity and 5% CO_2).

3.2.1 Proliferation assay for 3T3-Swiss albino cells

Cells were plated in 12-well-plates in RPMI 1640 containing 10% FCS and 1% penicillin-streptomycin at a density of 10^5 cells in one ml per well. Twenty four hour later, medium was replaced by a test medium (RPMI 1640™, 0.5% FCS and 1% penicillin-streptomycin) containing test samples (0 to 200 µl/ml SE, PE or PSE; 0 to 100 ng/ml porcine PDGF-R & D system, MN, Cat.#125PD). Maximal stimulation of proliferation was evaluated by adding 10% FCS in control wells. Each dose of each test sample was assayed in triplicate.

Two days later, cells were washed with RPMI 1640™, then trypsinized with 1 ml Trypsine/EDTA (Gibco, Cat.#25-300-013). Trypsinisation was stopped with 100 µl of pure FCS, cells in suspension were triturated, diluted. Finally, they were counted in a Coulter Counter ZM™ (Coulter Electronics of Canada, Ltd), equipped with a Coulter Channelyzer 256™, allowing for measurement of both cell number and cell average diameter.

In this cell line, both PE and SE stimulated cell proliferation in a dose-dependent manner between 1 and 100 µl of extracts. Combination of PE and SE was synergic on cell proliferation, that is the effect of PSE was more than the addition of the effects of PE and SE. This synergy was observed at the doses of 1 and 10 µl/ml, but not 100 µl/ml. In fact, the 100 µl/ml dose was less potent than the 10 µl/ml dose in all PSE tested. A dose response study from 0 to 200 µl/ml revealed that PSE induced a dose-related linear stimulation of cell proliferation from 0 to 10 µl/ml, the effect being progressively blunted between 50 and 200 µl/ml. Thus, the maximal effect on cell proliferation was estimated to be at 10-50 µl/ml of PSE.

3.3 M.T.T. Test

A quantitative colorimetric assay for porcine primary cultured fibroblasts (PPCF) and human foreskin fibroblasts (HFF) survival was developed in order to assess potential cytotoxicity of the extracts. The assay is dependent on the reduction of the tretrazolium bromide, from Sigma Chemical, St Louis, MO) by the mitrochondrial deshydrogenase of viable cells to form a blue formazan product. The assay was run in 96 well plates in DMEM/Ham F12 medium (Gibco). Cells were plated at confluency (4.0×10^4 cells/well) and the procedure was conducted exactly as described by Hansen M.B. et al. (J. Immunol. Methods, 1989, 119:203-210). All test samples (0 to 100 µl/ml) were tested in triplicate.

In order to determine if the inhibition of the proliferative effect observed at doses higher than 50 µl/ml was due to a cytotoxic effect, MTT tests were performed on a dose range (0 to 100 µl/ml) of PSE. As shown in Fig. 4, even high concentrations of PSE (50, 75, 100 µl) had no negative effect on cell survival, indicating an absence of cytotoxicity. In fact, an increase in optical density was noted at concentrations higher than 50 µl/ml, that reflected a good cellular health.

4. *IN VIVO* ASSAYS

4.1 Animals and wounds

Sprague-Dawley male rats (275-300g) were purchased from Charles River Canada Inc. (St Constant, Quebec). They were housed in individual cages and fed *ad libitum*. Each experimental group contained 7 to 8 rats. Wounds were performed as previously described by Garrel DR et al. (J. Surg. Res., 1991, 51:297-302). Briefly, one 6 cm-full-thickness cutaneous incision was

made longitudinally on the dorsal skin of each rat under pentobarbital anesthesia. The incisions were immediately closed with Dermalon 4.0™ sutures placed 1 cm apart.

4.2 Experimental treatments

Serum and platelet extract were prepared as described above. Except otherwise specified, they were applied onto the wounds in combination with a fibrin matrix purchased from Haemacure Biotech Inc. (Pointe-Claire, Québec). Platelet-Serum extracts were used to reconstitute a lyophilized thrombin powder (1 ml extract for 100 IU thrombin) in presence of calcium chloride (40 mM). This mixture was applied directly onto the wound in combination with a bovine fibrinogen solution (40-50 mg/ml) in picopure water. For a 6 cm-long wound, 250 µl of each preparation was used (total volume: 500 µl). The contact of the fibrinogen with thrombin in presence of calcium chloride resulted in an immediate polymerisation of the fibrinogen to fibrin, a solid matrix entrapping growth factor extracts and slowly releasing them into the wound. Several experiments were conducted to establish the efficacy and the optimal dose of extracts.

Experiment 1

The effect of fibrin matrix alone (500 µl) vs bovine serum albumine (BSA Sigma Chemical, St Louis, MO), 60 mg/ml as a control group was determined.

Experiment 2

The effect of growth factor extract (250 µl of PE, 10^{10} platelet equivalent/ml in 1% SE) alone (diluted to 500 µl in sterile saline) or in 500 µl fibrin matrix were determined. Control rats were treated with a solution of BSA, 60 mg/ml.

Experiment 3

The dose-effect relationship of growth factor extract (27,83 and 250 µl of PSE, 10^{10} platelet equivalent/ml in 1% SE) in 500 µl fibrin matrix, vs control (BSA, 60 mg/ml) was determined.

Experiment 4

The dose effect of growth factor extract (250, 500, 1000 µl of PSE, 10^{10} platelet equivalent/ml in 1% SE, lyophilized and reconstituted in 250 µl sterile water) in 500 µl fibrin matrix, vs control (BSA, 60 mg/ml) were determined.

4.3 Wound breaking strength

In all four experiments, wound breaking strength was measured 14 days postinjury. Initial experiments showed that the maximal effect of extracts was seen at that time. Rats were sacrificed by decapitation, the dorsal skin of each rat was excised and cut into 4 strips, 1 cm wide by 5 cm long, with razor blades positioned onto a rigid support. Haemorrhaged or infected wounds were discarded. Each strip was placed between the clamps of a 1101 Instron™ tensometer and the amount of force (Newtons) required to brake the strip was recorded. Tension was applied at 20 mm/min., and all measurements were done in a blind fashion.

The efficacy of PSE as a wound healing enhancer was tested in a model of rat surgical wound. Measured parameters included wound breaking strength 14 days post wounding, as well as histological examination of the scars.

As shown in Fig. 5 (experiments 1 and 2), the fibrin matrix or the PSE alone (200 µl) had no effect on wound strength. However, combination of PSE (200 µl) and fibrin matrix resulted in a 46% increase in wound strength. The dose-activity relationship was deduced

from experiments 3 and 4 (Fig. 6). In experiment 3, there was a dose-dependent increase in wound strength, from 0 to 250 µl of PSE (regression breaking strength/dose, r=0.51, P=0.013). Doses higher than 250 µl PSE failed to further increase wound breaking strength (experiment 4), and the 1 ml dose slightly reduced the stimulatory effect.

4.4 Histological evaluation

On the day of the sacrified one strip of each wound from experiment 2 was fixed in 10% formaldehyde, and later embedded in paraffin. Four-micrometer thick sections were stained with Hematein-Phloxin-Safran™ (HPS) and examined under light microscopy (Optiphot 2™ microscope, from Nikon).

Morphological evaluation includes aspect of the epidermis and of the cells within the scar, whereas morphometric evaluation includes thickness of the epidermis surrounding the scar, width of the scar at the level of both superficial and reticular dermis and cellularity, by counting fibroblasts per microscopic field, magnification x 750.

Finally, some 4 µm-thick sections were stained with Sirius Red F3BA™ and examined under polarized light to evaluate the aspect of collagen within the scar.

Histological evaluation of the scars was conducted on wounded skin samples from experiment 2. At the epidermis level, the epithelium surrounding the scar was pluristratified, fully differentiated and keratinized in all treatment groups. Moreover, epidermis thickness was identical in all treatment groups (Fig. 7). At the dermis level, most of the cells within the scar were fibroblasts surrounded by collagen. When stained with Sirius Red, collagen fibers

within the scar were shown to be unorganized and of smaller diameter when compared to those of normal dermis. Although scars did not differ qualitatively between treatment groups, scars from PSE/fibrin treated wound were wider and contained more cells than control and PSE alone treated wounds (Fig. 7). Positive significant correlations were found between wound strength and morphometric parameters of experiment 2 as illustrated in Table 1, wherein the coefficients of correlation were calculated from 23 observations.

Table 1

Correlation between morphometric parameters and wound strength

	CELLULARITY (cell number)	SCAR WIDTH (mm)
WOUND BREAKING STRENGTH (N)	r = 0.56 P = 0.005	r = 0.59 P = 0.004
CELLULARITY (cell number)		r = 0.68 P < 0.001

Thus, PSE increased wound breaking strength in vivo when administered in a fibrin matrix. This effect was associated with increased scar width and cellularity. In all observed histological sections, no sign of inflammation, of cheloïd or of tumoral foci could be detected.

In accordance with the present invention, there is described the manufacturing technology, the analytical content of one whole blood-derived growth factor extract, and its applications to wound healing when applied in combination with a fibrin matrix.

The association between a seric and a platelet extract yielded a preparation containing at least significant amounts of PDGF, TGFβ 2, IGF-I, II and IGFBPs. Although growth factors were only characterized by

immunoreactivity in accordance with one embodiment of the present invention, numerous reports confirm that platelets and serum are two major sources of growth factors.

Platelets α granules are the richest *in vivo* source of TGFβ and PDGF. Porcine platelets contain two isotypes, TGFβ 1 and β 2. Human and porcine TGFβ 1 have total sequence identity and TGFβ 2 has approximately 70% homology with TGFβ 1. All TGFβs are naturally found as a latent, high molecular weight, inactive complexes in platelets. In the extracts of the present invention, the measurement of non-extracted and acid-extracted platelet extracts yielded the same amount of TGFβ 1 and β 2, indicating that TGFβ 1 and β2 are present in our extracts in its active form of about 25 KD. Since activation of TGFβ in the latent complex has been shown to occur by treatment with acid, urea or heat *in vitro*, it was concluded that the heating step of the PE preparation was responsible for this activation.

PDGF is a disulfide-linked dimer with a molecular weight 30-32 KD. The subunits of the dimer are two related polypeptides designated the A and B chains. Although human platelet PDGF has been shown to consist of PDGF-AB and PDGF-BB, porcine platelet PDGF consists primarily of PDGF-BB homodimers. Because of its higher affinity for the type B PDGF receptor, PDGF-BB is more potent than PDGF-AB in stimulating cell proliferation *in vitro*. However, *in vivo* data suggest that PDGF AB and BB have equal potency as wound healing enhancers.

Both PDGF and TGFβs are known as stimulators of wound healing. They both induce extracellular matrix synthesis, granulation tissue formation and increase wound breaking strength in a variety of animal models. Both recombinant TGFβ 2 and PDGF-BB are currently

tested in clinical trials as therapeutic agents for the healing of chronic ulcers with promising initial results.

In addition to PDGF and TGFβ, platelet α granules have been shown to contain a number of agents that might play a role in the process of wound healing. Among these substances are Platelet Factor 4 (PF4), a platelet-derived Endothelial Cell Growth Factor (pdECGF), an Epidermal Growth Factor-like protein (EGF), and traces of IGF-I, IGF-II and IGFBP3.

Insulin-like growth factors I and II are two related peptides of approximately 7500 KD and of identical sequence in human and pig. In serum, they circulate in large amount, tightly bound to specific, high affinity binding proteins. IGF-I is the most important mediator of the biological actions of growth hormone and possesses strong systemic and local anabolic actions. Both IGFs and IGFBPs are present in wound fluid in the course of wound healing. When administered alone or in combination with IGFBPs, IGF-I has been shown to stimulate wound strength and granulation tissue formation in models of normal or impaired wound healing.

Thus, the analytical results showed that the process of extraction in accordance with the present invention successfully recovered IGFs and IGFBPs from serum, and PDGF and TGFβ from platelets. In addition, other bioactive substances, such like PF4, pdECGF or EGF might also be present and might participate in the biological activity of PSE.

The process of the present invention for the manufacture of PSE is new, simple and inexpensive. It involves a few number of steps that can be performed in less than 24 hours to produce a mixture made of at

least 5 important growth factors for wound healing (PDGF, TGFβ, IGF-I, IGF-II, IGFBPs).

Moreover, the porcine origin of the blood, in accordance with one embodiment of the present invention, guaranties: 1) a total absence of potential transmission of human viral agents (hepatitis, HIV, etc.); 2) the production of growth factors nearly 100% identical to their human counterparts and, 3) a reliable, largely available and highly reproducible source of raw material.

The *in vitro* and *in vivo* studies showed that the platelet/serum extracts of one embodiment of the present invention have positive effects on wound healing, mediated by at least two different mechanisms, cell proliferation and collagen synthesis.

In cell culture, both the platelet and serum extracts of the present invention stimulated fibroblast proliferation in a dose dependent manner, and coadministration of both extracts resulted in a synergic effect. Since SE and PE contained IGFs and PDGF, respectively, it is suggested that the synergy was due to interactions between these two growth factors. This synergy was first reported in cultured 3T3 fibroblasts, then confirmed *in vivo* in animal models of wound healing. It has been explained by the complementary roles of PDGF and IGFs in the cell cycle, PDGF being a competence factor and IGFs being a progression factor.

PSE induced a dose-dependent increase of porcine primary cultured fibroblasts (PPCF) or 3T3 up to 10 µl/ml, while the stimulatory effect was inhibited at doses equal or above 50 µl/ml. In parallel, PSE was not cytotoxic at doses up to 100 µl/ml and stimulated protein (collagen) synthesis in a dose dependent fashion between 0 and 100 µl/ml. Based on these results, the hypothesis of a dual mode of action for PSE was

raised: at low dose (0-10 µl/ml), it would stimulate preferentially fibroblast proliferation; at higher doses (≥ 50 µl/ml), the effect on extracellular matrix synthesis would be predominant and would overcome that on cell proliferation.

In vivo studies were conducted using a fibrin matrix as carrier. PSE alone failed to increase wound breaking strength, whereas PSE in fibrin did. This indicated that the fibrin sealant was necessary to maintain growth factors in contact with the wound for a sufficient time. This type of matrix is safe, natural and biodegradable. No sign of fibrin was detected by histological observation 14 days post administration, suggesting that the fibrinolysis was complete at that time. When applied in combination with the fibrin glue, the PSE of the present invention stimulated wound breaking strength in a surgical model in a dose dependent fashion from 0 to 250 µl of extract, with no further increase at higher doses. Histological observation of the treated wound revealed increased cellularity and wider scars. This suggested that the increase of wound strength was due to an increased number of fibroblasts, synthetising larger amounts of collagen. Moreover, the absence of inflammation, of transformed cells and of cheloïds indicated that the treatment was safe.

These *in vitro* and *in vivo* studies suggest that the platelet/serum extract of the present invention is a powerful stimulator of granulation tissue formation. Its potential therapeutical applications include the treatment of chronic ulcers and surgical wounds, and the regeneration of injured soft tissues.

According to the *in vitro* and *in vivo* results, SE and PE of the present invention might be used alone or advantageously in combination (PSE) because they

work synergically. *In vivo*, they have to be administered topically in a matrix slowly releasing them, such as fibrin glue, collagen, polymers among others.

If fibrin is selected as a carrier, for a 6 cm long surgical wound, the bioactive formulation is 50 to 1000 µl of a preparation made of 10 to 1000 IU/ml thrombin, 20 to 200 mg/ml fibrinogen, and 50 to 500 µl/ml SE (0.1 to 5%), PE (10^8 to 10^{12} platelet equivalent/ml buffer) or PSE (10^8 to 10^{12} platelet equivalent/ml SE 0.1 to 5%) in presence of $CaCl_2$ (1 to 100 mM final).

Recently, a randomized, double blind and placebo clinical study reported significant improvements in diabetic ulcers treated with an homologous human platelet lysate derived from pooled human platelets. Moreover, a cost-efficacy analysis conducted for this study revealed that the treatment was associated with a 38% decrease in the medical costs when compared to a conventional therapy. Although the composition of the PSE differs from that of the human platelet lysate (thrombin activated platelet wound healing formula) developed by Curative Technologies Inc., the results of this study illustrate the therapeutical potential and the advantages of growth factor extracts over recombinant growth factors.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Preparation of Platelet Extract

Three liters of platelet-rich-plasma (PRP) having a platelet concentration of 464 X 10^9 platelets/L was used. The amount of graphite to add was determined using the following formula:

$$Q = K.D_X\ [PRP].\ V.\ 10^{-9}$$

where K is an empirically determined constant of 0.082, D_X is a ratio of the density of the neutral support with respect to the density of carbon (1 for graphite), [PRP] is the platelet concentration per liter of PRP (464 X 10^9), V is the PRP volume in L (3), and Q is the amount of graphite to add in g, thus Q=114 g of graphite.

This graphite is previously washed in water and carefully mixed with the three liters of PRP. After a 2 min. mixing, the platelet-adsorbed-graphite mixture is filtered under atmospheric pressure through a especially design high capacity filtering membrane.

After 10 min. of filtration, the adsorbed platelet are washed twice with three liters of phosphosaline buffer pH 7.4, then the washed filtrate is transferred in a beaker. The membrane is rinsed with 1 L of distilled water and the temperature of the washed filtrate in the beaker is raised to 100°C for 1 min.

The washed filtrate in the beaker is filtered and/or centrifuged; the supernatant is lyophilized. The lyophilisate constitutes the solid platelet extract (PE) which can be reconstituted.

In vitro bioassays were performed using porcine primary cultured fibroblasts (PPCF) and 3T3-Swiss albino cells (3T3) as previously described in sections 3.1, 3.1.1, 3.2, 3.2.1 and 3.3 of the description.

Results

The following were measured according to the procedure described above in the disclosure.

PDGF = 2.0 µg/l

$TGF\beta_1$ = 0.0 µg/l

$TGF\beta_2$ = 5.6 ng/l

IN VITRO BIOASSAYS

		Proliferation assay (cell count)			M.T.T. test (d.o. 570 nm)		
Cell type	Dosage	Mean	S.E.M.	% proliferation	Mean	S.E.M.	% oxydation
PPCF	control	75858	763	100	0.21	0.017	100
	1 ul/ml	83998	3239	111	0.23	0.035	111
	10 ul/ml	102259	1855	135	0.26	0.025	126
	100 ul/ml	141723	3416	187	0.29	0.038	140
3T3	control	220900	3186	100			
	1 ul/ml	230704	10642	104			
	10 ul/ml	284342	937	129			
	100 ul/ml	281076	12321	127			

EXAMPLE II

Preparation of Platelet Extract

The procedure as described in Example I was carried out, except that carbure of silicium was used instead of graphite. This material has a density constant of 3.23, which resulted in a Q value of 335g of carbure of silicium used.

EXAMPLE III

Preparation of Platelet Extract

The procedure as described in Example I was carried out, except that nitrure of silicium was used instead of graphite. This material has a density constant of 3.44, which resulted in a Q value of 357g of nitrure of silicium used.

EXAMPLE IV

Preparation of Platelet Extract

Procedure

Three liters of platelet-rich-plasma (PRP) having a platelet concentration of 464 X 10^9 platelets/L was used. The amount of carbon to add was determined using the following formula:

$$Q = K[PRP]V$$

where K is an empirically determined constant of 0.082, [PRP] is the platelet concentration per liter of PRP (464 X 10^9), V is the PRP volume in L (3), and Q is the amount of carbon to add in g, thus Q=114 g of carbon. This carbon is previously washed in 500 ml of picopure water, agitated vigorously and then centrifuged for 2 min. at 2000g using a Rotor™ JA10 (Beckman™ J2-21).

The precipitated carbon is kept and carefully mixed with the three liters of PRP. After a 2 min. mixing, the platelet-adsorbed-carbon mixture is filtered through a Whatman filter paper 2V™. The filtered PPP is discarded.

After the filtration, the adsorbed platelet are washed twice with 450 ml of phosphosaline buffer pH 7.4, then the washed filtrate is transferred in a beaker. The adsorbed platelet are resuspended in picopure water (>18.2 megaohms) to obtain a final concentration of 10^9 pq/ml and the temperature of the beaker is cooled to about 0-4°C using an ice bath.

The adsorbed platelet are subjected to a first ultra-sound treatment with a sonicator (Kontes™ 40-watt; output control:60; monitor scale:20) for about 45 sec., a pause of 30 sec. and a second ultra-sound treatment for about 45 sec.

The washed filtrate in the beaker is centrifuged for 5 min. at 5000g; the precipitate is resus-

pended in a minimum quantity of picopure water and centrifuged again under the same conditions. Both supernatants are combined, frozen in liquid nitrogen and then lyophilized. The lyophilisate constitutes the solid platelet extract (PE) which is reconstituted in picopure water (>18.2 megaohms) to obtain a final concentration of 10^{10} pq/ml, centrifuged for 30 min. at 10,000g. The supernatant is filtered first through 0.8 µm and 0.2 µm filters (Nalgene, Rochester, NY). The preparation is aliquoted and kept at -80°C.

In vitro bioassays were performed using porcine primary cultured fibroblasts (PPCF) and 3T3-Swiss albino cells (3T3) as previously described in sections 3.1, 3.1.1, 3.2, 3.2.1 and 3.3 of the description.

Results

The following are measured according to the procedure described above in the disclosure.

PDGF = 4.5 µg/l

$TGF\beta_1$ = 0.870 µg/l

$TGF\beta_2$ = 46.0 ng/l

***IN VITRO* BIOASSAYS**

		Proliferation assay (cell count)			M.T.T. test (d.o. 570 nm)		
Cell type	Dosage	Mean	S.E.M.	% proliferation	Mean	S.E.M.	% oxydation
PPCF	control	76982	1706	100	0.21	0.017	100
	1 ul/ml	84941	1658	110	0.26	0.025	122
	10 ul/ml	103249	3076	134	0.27	0.013	130
	100 ul/ml	205656	12731	267	0.41	0.033	195
3T3	control	225163	5149	100			
	1 ul/ml	239822	878	107			
	10 ul/ml	268620	7321	119			
	100 ul/ml	446137	5564	198			

EXAMPLE V

Preparation of Platelet Extract

Procedure

The procedure as described in Example IV is carried out, except that activated carbon Darco™ G-60 (Aldrich) was used instead of carbon.

In vitro bioassays were performed using porcine primary cultured fibroblasts (PPCF) and 3T3-Swiss albino cells (3T3) as previously described in sections 3.1, 3.1.1, 3.2, 3.2.1 and 3.3 of the description.

Results

The following are measured according to the procedure described above in the disclosure.

PDGF = 3.16 µg/l

$TGF\beta_1$ = 0.873 µg/l

$TGF\beta_2$ = 5.8 ng/l

IN VITRO BIOASSAYS

		Proliferation assay (cell count)			M.T.T. test (d.o. 570 nm)		
Cell type	Dosage	Mean	S.E.M.	% proliferation	Mean	S.E.M.	% oxydation
PPCF	control	73117	2345	100	0.21	0.017	100
	1 ul/ml	68599	2354	94	0.22	0.014	107
	10 ul/ml	75689	4188	104	0.21	0.023	102
	100 ul/ml	159688	5937	218	0.41	0.039	194
3T3	control	237384	5019	100			
	1 ul/ml	246642	9229	104			
	10 ul/ml	256782	7338	108			
	100 ul/ml	427903	2352	180			

EXAMPLE VI

Preparation of Platelet Extract

Procedure

Three liters of platelet-rich-plasma (PRP) having a platelet concentration of 300 to 500 X 10^9 platelets/L are centrifuged for about 20 min. at 1500g (BRAKE=6; Rotor™ JA10; Beckman™ J2-21). The precipitated platelets are resuspended in phosphosaline buffer (PBS) to obtain a final concentration of 10^9 platelet equivalent per ml (pq/ml), which corresponds to the first washing step.

The resuspended platelets are centrifuged for a second time at the conditions mentioned above. The precipitated platelets are resuspended in picopure water (>18.2 megaohms) to a final concentration of 10^{10} platelet equivalent per ml (pq/ml).

The solution is cooled to about 0 to about 4°C in a ice bath and is subjected to a shock treatment consisting of two ultra-sound treatment with a sonicator (Kontes™ 40-watt; output control: 60; monitor scale: 20) for 45 sec. with a pause of 30 sec. in between.

The treated solution is frozen using liquid nitrogen and lyophilized overnight. The lyophilized platelets are reconstituted in picopure water to obtain a final concentration of 10^{10} pq/ml. The membrane fragments are eliminated by ultracentrifugation or by filtration through 0.8 µm, 0.45 µm and 0.2 µm filters (Nalgene, Rochester, NY). The preparation is aliquoted and kept at -80°C.

In vitro bioassays were performed using porcine primary cultured fibroblasts (PPCF) and 3T3-Swiss albino cells (3T3) as previously described in sections 3.1, 3.1.1, 3.2, 3.2.1 and 3.3 of the description.

Results

The following are measured according to the procedure described above in the disclosure.

PDGF = 14.2 µg/l

$TGF\beta_1$ = 3.9 µg/l

$TGF\beta_2$ = 26.7 ng/l

***IN VITRO* BIOASSAYS**

		Proliferation assay (cell count)			M.T.T. test (d.o. 570 nm)		
Cell type	Dosage	Mean	S.E.M.	% proliferation	Mean	S.E.M.	% oxydation
PPCF	control	74174	1644	100	0.18	0.009	100
	1 ul/ml	80234	4050	108	0.24	0.023	130
	10 ul/ml	113888	1062	154	0.35	0.037	191
	100 ul/ml	219769	7233	296	0.34	0.010	184
3T3	control	208538	5320	100			
	1 ul/ml	229108	7577	110			
	10 ul/ml	269219	9914	129			
	100 ul/ml	388493	10111	186			

EXAMPLE VII

Preparation of Serum Extract

A 300 ml of whole serum was first acidified with 18.2 ml of formic acid (88% formic acid, Fisher Scientific, Neapan, Ontario) at room temperature. After 5 min. of agitation at room temperature, 700 ml of pure acetone was added. The agitation was continued for another 5 min., then the suspension was filtered through a Whatman filter paper 2V™.

The filtrate was collected and slowly transferred in a beaker containing 1400 ml of pure acetone constantly agitated. The order in the addition is of extreme importance.

The precipitate is filtered through a Whatman filter paper 2V™, washed three times with 500 ml of pure acetone, then three times with 500 ml of ether, then dried. The solid mass is 1.7 g and is reconstituted in 170 ml of picopure water (18.2 Ohm) (1% w/v), filter-sterilized through 0.22 µm filters (Nalgene, Rochester, NY) and aliquoted under laminar flux (Canadian Cabinets Co, Ltd., V6 MW99.C30™). This solution of pH 6.5 and 1% of final concentration, constitutes the serum extract (SE).

EXAMPLE VIII

Preparation of Serum Extract

Procedure

A 300 ml of whole serum was centrifuged for 15 min. at 3000g, the supernatant is kept and centrifuged for a second time under the same conditions. The centrifuged supernatant is acidified at pH 2 with 88% formic acid (Fisher Scientific, Neapan, Ontario) at room temperature.

The acidified serum is cooled at 4°C in a cold room. A quantity of 75% acetone is added and agitated magnetically for an hour to precipitate the proteins. The suspension is filtered through a Whatman filter paper 2V™.

The filtrate was collected and slowly transferred in a beaker containing an amount of pure acetone to obtain a final concentration of 90% acetone. After an incubation of one hour, the suspension is filtered through a Whatman filter paper 2V™.

The precipitate is washed twice with pure acetone, and then a third time with anhydride ether, then dried using a lyophilizer.

The solid mass is reconstituted in picopure water (>18.2 megaohms) to obtain a final concentration of 1%, filter-sterilized through 0.22 µm filters

(Nalgene, Rochester, NY) and aliquoted under laminar flux (Canadian Cabinets Co, Ltd., V6 MW99.C30™) and kept at -80°C.

In vitro bioassays were performed using porcine primary cultured fibroblasts (PPCF) and 3T3-Swiss albino cells (3T3) as previously described in sections 3.1, 3.1.1, 3.2, 3.2.1 and 3.3 of the description.

Results

The following are measured according to the procedure described above in the disclosure.

IGFII = 47.9 µg/l

IGFI = 16.9 µg/l

$TGF\beta_1$ = 1.98 µg/l

$TGF\beta_2$ = 0.027 µg/l

***IN VITRO* BIOASSAYS**

		Proliferation assay (cell count)			M.T.T. test (d.o. 570 nm)		
Cell type	Dosage	Mean	S.E.M.	% proliferation	Mean	S.E.M.	% oxydation
PPCF	control	74087	1761	100	0.21	0.017	100
	1 ul/ml				0.20	0.006	93
	10 ul/ml				0.17	0.007	84
	100 ul/ml	61368	3120	83	0.11	0.006	54
3T3	control	266384	1062	100			
	1 ul/ml	296206	5188	111			
	10 ul/ml	432394	1906	162			
	100 ul/ml	507700	18724	191			

EXAMPLE IX

Preparation of Serum Extract

Procedure

The procedure as described in Example VIII is carried out, except that the acidified serum is cooled at 0°C instead of 4°C. Also, the first precipitation using acetone is conducted such that the acetone is

added dropwise until a final acetone concentration of 72% is achieved, the second acetone addition is effected under the same conditions until a final acetone concentration of 75% is achieved, and a third acetone addition is effected under the same conditions until a final acetone concentration of 90% is achieved.

Results

The following are measured according to the procedure described above in the disclosure.

IGFII = 356 µg/l

IGFI = 88 µg/l

$TGF\beta_1$ = 21.5 µg/l

$TGF\beta_2$ = 0.290 µg/l

IN VITRO **BIOASSAYS**

		Proliferation assay (cell count)			M.T.T. test (d.o. 570 nm)		
Cell type	Dosage	Mean	S.E.M.	% proliferation	Mean	S.E.M.	% oxydation
PPCF	control	74437	2317	100	0.21	0.017	100
	1 ul/ml				0.17	0.017	83
	10 ul/ml				0.16	0.008	77
	100 ul/ml	67771	3727	91	0.16	0.007	74
3T3	control	260270	4083	100			
	1 ul/ml	310622	16617	119			
	10 ul/ml	391146	6134	150			
	100 ul/ml	449006	6863	173			

EXAMPLE X

Preparation of Serum Extract

The procedures as described in Example VII and VIII are carried out, except that formic acid is replaced by the following acid mixture, trifluoroacetic acid, chlorydric acid and phosphorous acid.

EXAMPLE XI

Preparation of Final Extract

The PE of Example I, II, III, IV, V or VI is reconstituted with SE of Example VII, VIII, IX, or X to obtain a final concentration of 10^{10} platelet per ml without neutralization. The mixture is centrifuged at 10,000g for 15 min. at +4°C, then filter sterilized through 0.22 µm filters and aliquoted under laminar flux.

EXAMPLE XII

Preparation of Final Extract

The PE of Example I, II, III, IV, V or VI is reconstituted with SE of Example VII, VIII, IX, or X to obtain a final concentration of 10^{10} platelet per ml. The mixture is neutralized using a Amberlite IRA-400(OH)™ resin, centrifuged at 10,000g for 15 min. at +4°C, then filter sterilized through 0.22 µm filters and aliquoted under laminar flux.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. A process for the preparation of platelet-extract, which comprises the steps of:

a) concentrating a platelet-rich-plasma prepared from whole blood by passing through a neutral support for adsorbing the platelets or by centrifuging whole blood at about 1000g to about 3000g; and

b) releasing the concentrated platelets of step a) by ultra-sound shock.

2. A process for the preparation of serum-extract, which comprises the steps of:

a) precipitating a concentrated plasma prepared from whole blood by passing through a neutral support for adsorbing the platelets or by centrifuging whole blood at about 1000g to about 3000g, a platelet-poor-plasma or a serum by adding an amount of acetone solution to obtain an acetone concentration of about 68 to about 95% at a temperature of about -15 to 5°C; thereby obtaining a solid plasma or serum precipitate; and

b) the serum precipitate of step a) is reconstituted in an appropriate buffer to obtain a serum-extract of a concentration of about 0.5 to 6% of protein weight/volume.

3. A process for the preparation of platelet-serum-extract, which comprises the steps of:

a) concentrating a platelet-rich-plasma prepared from whole blood by passing through a neutral support for adsorbing the platelets or by cen-

trifuging whole blood at about 1000g to about 3000g;

b) releasing the concentrated platelets of step a) by ultra-sound shock;

c) precipitating the concentrated plasma of step a), a platelet-poor-plasma or a serum by adding an amount of acetone solution to obtain an acetone concentration of about 68 to about 95% at a temperature of about -15 to 5°C; thereby obtaining a solid plasma or serum precipitate;

d) the plasma or serum precipitate of step c) is reconstituted to a concentration of about 0.5 to 6% of protein weight/volume and mixed to the released platelets of step b) to obtain a platelet-serum-extract of a concentration which substantially has a physiological concentration to give a maximum synergy in promoting wound healing.

4. The process of claim 3, wherein the precipitation step c) is conducted using a first amount of acetone solution to obtain an acetone concentration of about 68% to about 72% and the resulting supernatant is precipitated with a second amount of acetone solution to obtain an acetone concentration of about 84% to about 95%, wherein the second precipitate is collected to form the serum precipitate.

5. The process of claim 4, wherein the plasma or serum precipitate of step c) is reconstituted in an appropriate buffer.

6. The process of claim 3, wherein the neutral support of step a) is selected from the group consist-

ing of carbon, activated carbon, graphite and charcoal, SiC and Si_3N_4.

7. The process of claim 6, wherein the amount of neutral support used in step a) is determined by the following formula:

$$Q = K.D_x\ [PRP].\ V.\ 10^{-9}$$

wherein:

K is an empirically determined constant ranging from 0.010 to 1.000;

D_x is a ratio of the density of the neutral support with respect to the density of carbon;

[PRP] is the platelet concentration per liter of PRP;

V is the PRP volume in L; and

Q is the amount of neutral support in g.

8. The process of claim 7, wherein k is 0.082.

9. The process of claim 3, wherein the ultra-sound shock of step b) consists of two ultra-sound treatment with a sonicator for about 5 sec. to 10 min. with a pause of at least 30 sec. in between.

10. The process of claim 3, wherein after step a) a further step is added, which comprises:

a') filtering the adsorbed platelet-support mixture through a filtering membrane.

11. The process of claim 3, wherein said whole blood is of human, porcine, bovine, ovine or caprine origin.

12. A pharmaceutical composition for promoting wound healing, which comprises an effective concentra-

tion of platelet-extract prepared according to the process of claim 1 in association with a pharmaceutically acceptable carrier.

13. A pharmaceutical composition for promoting wound healing, which comprises an effective concentration of serum-extract prepared according to the process of claim 2 in association with a pharmaceutically acceptable carrier.

14. A pharmaceutical composition for promoting wound healing, which comprises an effective concentration of platelet-serum-extract prepared according to the process of claim 3 in association with a pharmaceutically acceptable carrier.

15. The pharmaceutical composition of claim 14, wherein said platelet-serum-extract concentration is between 10^8 to 10^{12} platelet equivalent/ml in combination with 0.5 to 2% serum-extract/ml.

16. The pharmaceutical composition of claim 14, wherein said pharmaceutically acceptable carrier is selected from the group consisting of fibrin glue, collagen, collagen derivatives, polysaccharidic polymers, gelatins, ointments and solid dressings suitable for wound treatment.

17. Use of the composition of claim 14 for the promotion of wound healing of a patient.

18. The method of use of the platelet-serum-extract prepared according to the process of claim 3 for promoting wound healing of a patient.

19. Use of the composition of claim 14 for the production of a medicament for the promotion of wound healing of a patient.

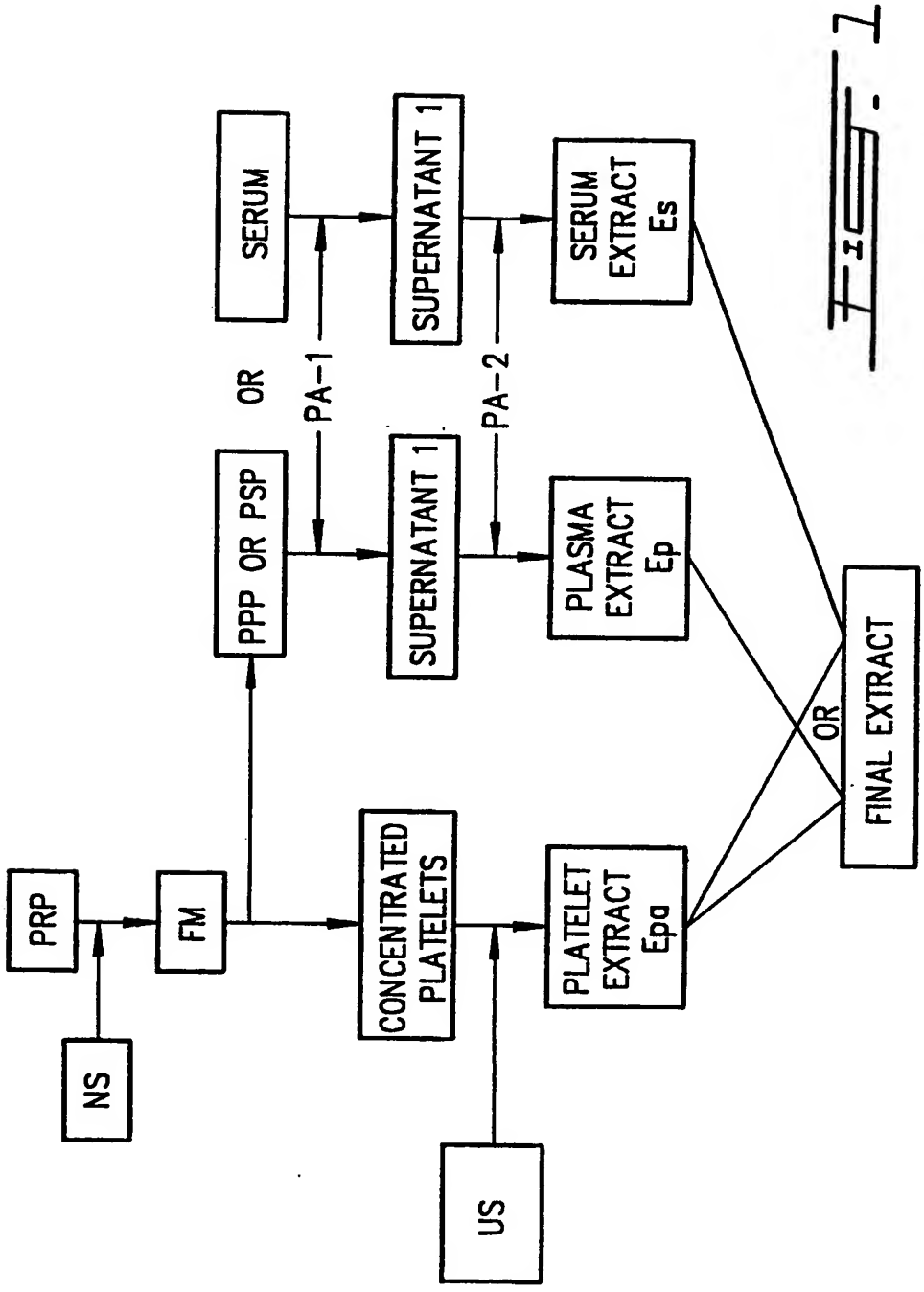

PRP
NS
FM
PPP OR PSP
SERUM
OR
PA-1
SUPERNATANT 1
PA-2
SUPERNATANT 1
CONCENTRATED PLATELETS
US
SERUM EXTRACT Es
PLASMA EXTRACT Ep
PLATELET EXTRACT Epa
OR
FINAL EXTRACT

Fig. 1

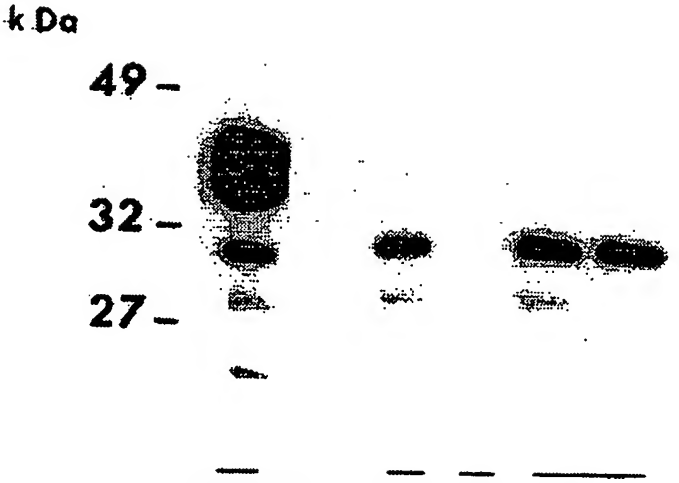


Fig. 2

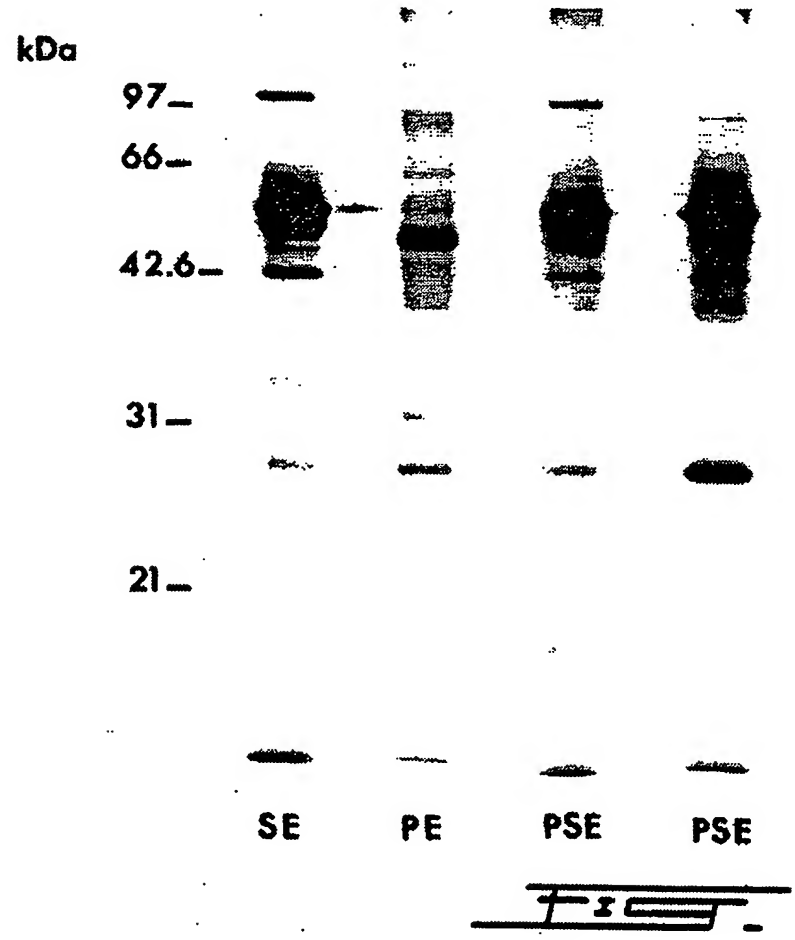


Fig. 3

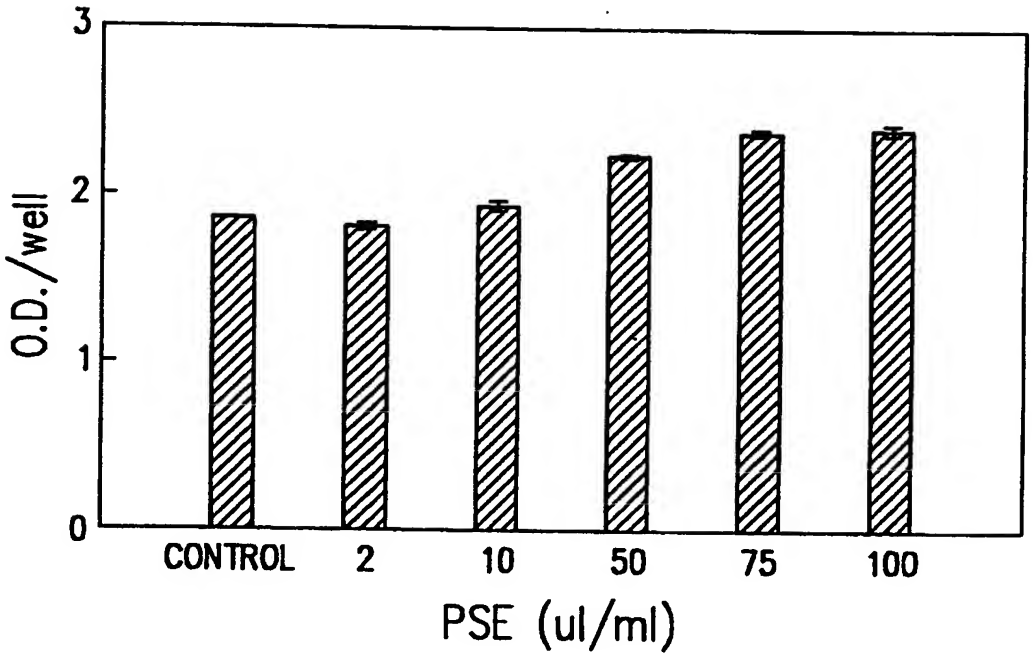


Fig. 4

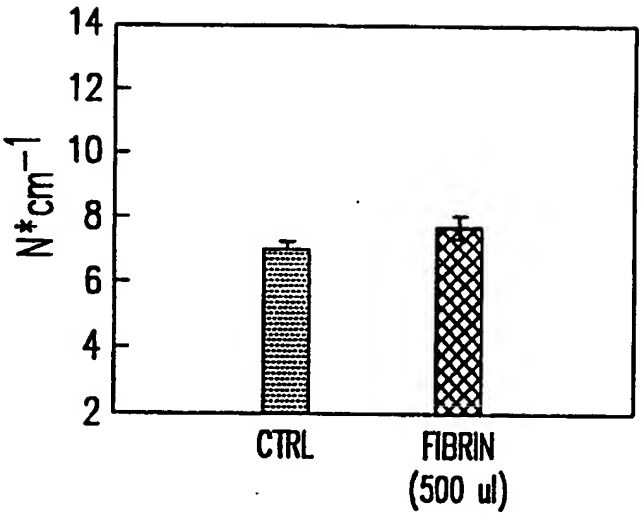
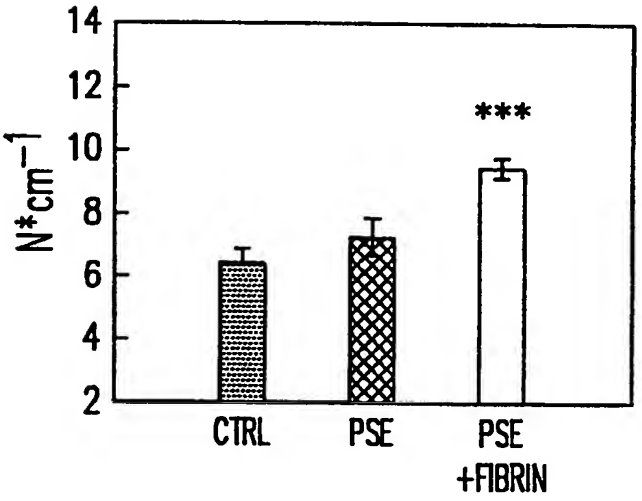
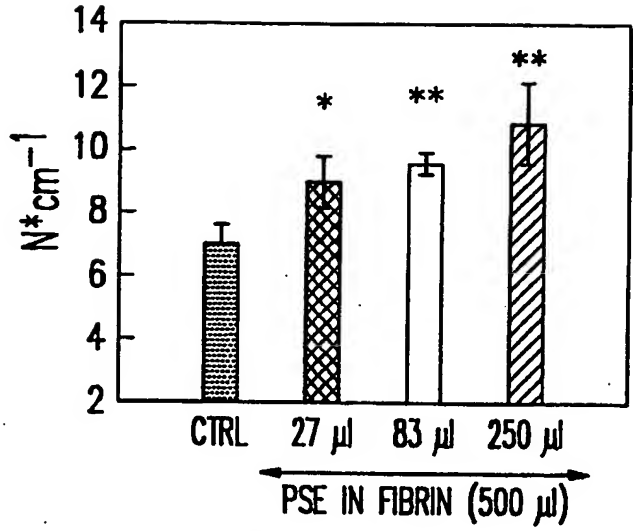


Fig. 5a



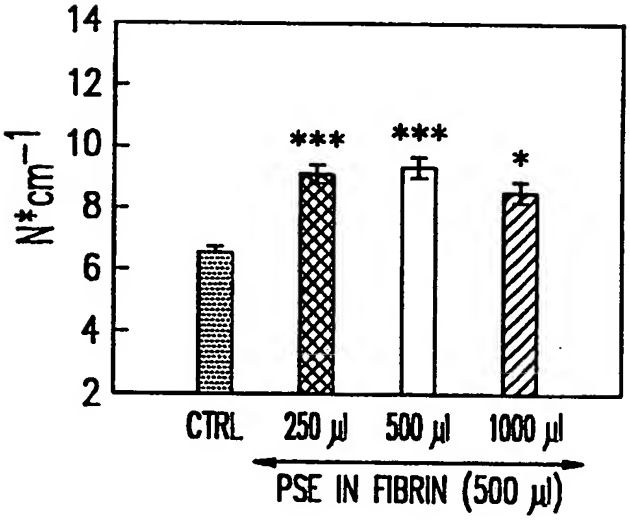
* p<0.05 WHEN COMPARED TO CONTROL.
** p<0.02 WHEN COMPARED TO CONTROL.
*** p<0.001 WHEN COMPARED TO CONTROL.

Fig. 5b



* p<0.10 WHEN COMPARED TO CONTROL.
** p<0.02 WHEN COMPARED TO CONTROL.
*** p<0.001 WHEN COMPARED TO CONTROL.

Fig. 6a



* p<0.10 WHEN COMPARED TO CONTROL.
** p<0.02 WHEN COMPARED TO CONTROL.
*** p<0.001 WHEN COMPARED TO CONTROL.

Fig. 6b

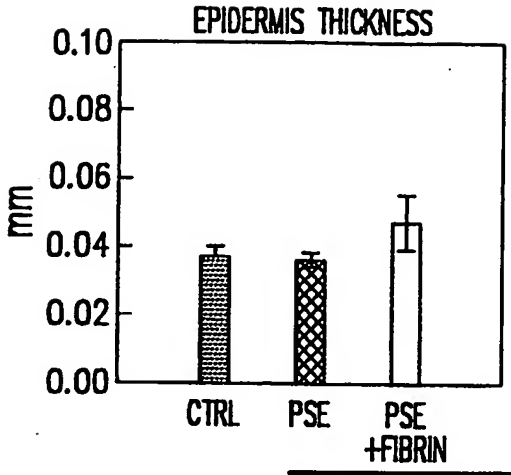
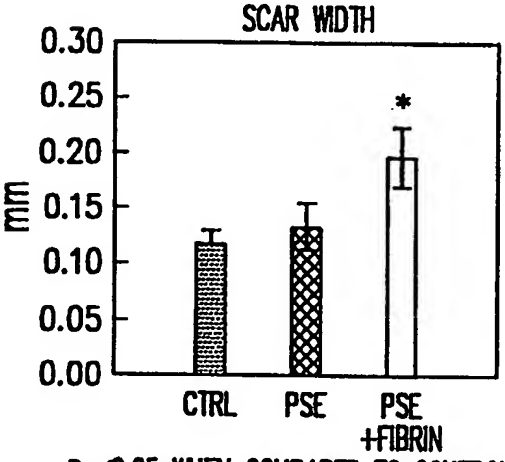
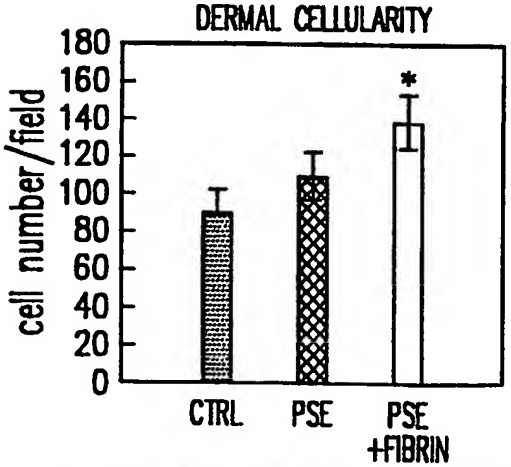


Fig. 7a



* p <0.05 WHEN COMPARED TO CONTROL.

Fig. 7b



* p <0.05 WHEN COMPARED TO CONTROL.

Fig. 7c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 94/00601

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/14 A61K35/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FILE SERVER STN KARLSRUHE,FILE MEDLINE ABSTRACT NO.94074153 & CLIN ENDOCRINOL (OXF), (1993 NOV) 39 (5) 583-9 ABRIBAT ET AL:'INSULIN-LIKE GROWTH FACTOR-I BLOOD LEVELS IN SEVERELY BURNED PATIENTS:EFFECTS OF TIME POST INJURY,AGE OF PATIENT AND SEVERITY OF BURN' see abstract ---	2,13
X	US,A,3 953 290 (UTHNE ET AL) 27 April 1976 see column 1, line 5 - column 2, line 54 ---	2,13
X	EP,A,0 136 093 (SAKAGAMI) 3 April 1985 see page 1, line 2 - page 4, line 4; example 1 ---	2,13
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 February 1995	22 -02- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 94/00601

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 8344, Derwent Publications Ltd., London, GB; Class B04, AN 83-804027 & JP,A,58 159 419 (FUJI ZOKI SEIYAKU) 21 September 1983 see abstract	2
A	CHEMICAL ABSTRACTS, vol. 116, no. 11, 16 March 1992, Columbus, Ohio, US; abstract no. 102145g, SEDGWICK ET AL 'EFFECT OF PROTEIN PRECIPITATING AGENTS ON THE RECOVERY OF PLASMA FREE AMINO ACIDS' page 360 ;column 1 ; see abstract & CAN.J.ANIM.SCI., vol.71, no.3, 1991 pages 953 - 957	2
A	WO,A,86 03122 (CURATECH,INC.) 5 June 1986 see page 2, line 28 - page 4, line 22	1,12
A	CHEMICAL ABSTRACTS, vol. 100, no. 7, 13 February 1984, Columbus, Ohio, US; abstract no. 49215w, WILLIAMS ET AL 'INTERACTIONS OF ULTRASOUND WITH PLATELETS AND THE BLOOD COAGULATION SYSTEM' page 387 ;column 1 ; see abstract & ULTRASOUND INTERACT.BIOL.MED.,(PROC.INT.SYMP.) 1980, 1983 pages 171 - 177	1

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA94/00601

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 17 and 18 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA 94/00601

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